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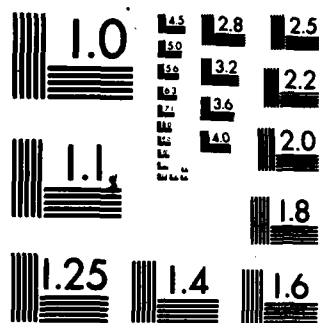
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CHARACTERIZATION OF THE CHEMICAL CONSTITUTION AND  
PROFILE OF PHARMACOLOGICAL ACTIVITY OF PGB<sub>x</sub>

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College of Pharmacy

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A. M. Burkman, R. W. Doskotch and D. D. Miller

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### The pharmacology of PGBx

The pharmacological studies conducted over the past four years have attempted to answer the following questions:

- (a) Does PGBx produce organ level effects that are expressions of PGBx's established mitochondrial actions?
- (b) Are the organ level effects related to PGBx's ability to act as a calcium ionophore?
- (c) In assessing its potential therapeutic utility, are there other actions of PGBx that are relevant?
- (d) Can an organ level bioassay be designed that is adaptable to the evaluation and comparison of biological activity of PGBx analogs and components?

The results of these studies are summarized in the pages that follow.

- 1) Using isolated, blood-perfused canine cardiac tissues subjected to ischemic damage, PGBx administered as a premedicant, produced a statistically significant degree of protection. The benefit which is dose-dependent, however, is so small as to be of questionable biological significance (see Technical Report No. 3, p. 1, submitted 26 February 1982).
- 2) The comparative effects of ischemia (30 min deprivation of blood supply) and calcium entry blockade were examined in isolated canine sinoatrial node (SAN), atrial ventricular node (AVN) and papillary muscle (PM) preparations cross circulated with blood from supporting dogs. Automaticity of SAN and AVN exhibited a high degree of resistance to ischemia, whereas both automaticity and tension development of PM were more susceptible and were ultimately abolished during the test period. Upon resumption of blood

supply, contractility rapidly returned to pre-ischemic levels. However, force-frequency analyses revealed that even after reperfusion, PM had lost the ability to respond normally to stimulus frequencies greater than 2 Hz. The calcium channel blocker, SKF 24260, exhibited little effect on PM automaticity, but greatly depressed PM contractility and SAN automaticity, leading to SAN arrest. It is suggested that acute deprivation of blood supply does not predominantly damage the functions of those tissues in which the slow inward current is initiated by calcium ions (See Appendix A). It therefore seems unlikely that a calcium iontophore (such as PGBx) would exert a profound protective effect on the performance of ischemic myocardium.

- 3) Median lethal doses of PGBx were estimated when administered to mice via intraperitoneal and intravenous routes. The incidence of lethality (and therefore the LD50) was time dependent over a 96-hour period. The animals were responsive and took nourishment during the 4-day post-injection period so that they apparently died from the direct effects of PGBx (or its metabolites) and not as a consequence of depression-induced dehydration or starvation. The 96-hour LD50's ( $\pm$  SE) for the IP and IV routes were  $90 \pm 27$  and  $69 \pm 6$  mg/kg, respectively (See appendix B).
- 4) While characterizing the cardiovascular pharmacodynamics of PGBx, the actions of its precursor PGB<sub>1</sub> were also examined. It has been generally assumed that prostaglandins of the B series are devoid of activity on the cardiovascular system and thus activities associated with PGBx are unique to this product. It was observed, however, that PGB<sub>1</sub> exerts a

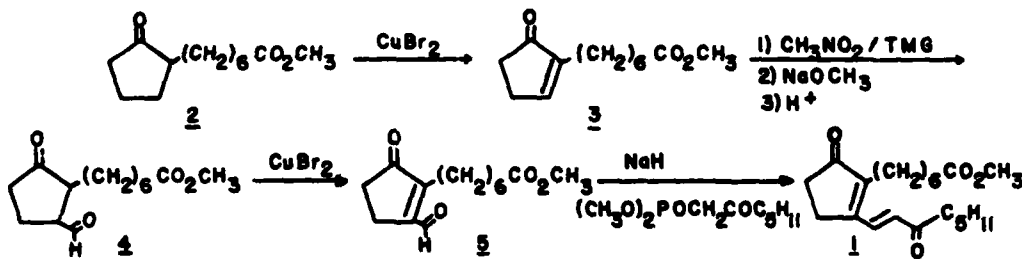
peripherally mediated increase in peripheral vascular resistance (See Appendix C). No such effect can be seen with PGBx. In fact PGBx tends to reduce peripheral vascular resistance (See Technical Report No. 2 p. 6, submitted 23 February 1981).

- 5) An examination of the effects of varying concentrations of PGBx on cardiac glycoside (ouabain) - induced, calcium dependent cardiotoxicity, in vivo, revealed that PGBx (10 mg/kg) exerted a protective effect and the concentrations of ouabain needed to induce cardiac arrhythmia and ventricular fibrillation were elevated. The concentration needed to provoke cardiac arrest was also elevated although significance at the 5% level could not be attained. This protective effect is not consistent with a calcium ionophore mechanism and could be interpreted as an activity operating by a mechanism unrelated to calcium disposition (see Appendix D).
6. An organ level biological assay was developed that can serve as an alternative to the mitochondrial assay and that may more closely reflect activities that are therapeutically relevant. The bioassay is based on the ability of PGBx to increase the contractility-provoking effect of a fixed concentration of (-) - isoproterenol in isolated right and left atria. The original assay was developed using the spontaneously beating guinea pig right atrium but subsequent attempts to refine the assay revealed that the electrically paced left atrium was a more reliable system and inotropic behavior could be monitored without chronotropic interference (see Technical Report No. 2, p. 4 submitted 23 February 1981).

### Studies on the Synthesis of PGB<sub>x</sub>

In the final year of this project we have continued to investigate new methods for the preparation of the methyl ester of 15-keto PGB<sub>1</sub> (1). Our desire was to find a pathway that was much shorter than the synthetic pathways that are currently available in the literature. We have reported previously on our synthetic scheme utilizing dimethyl 3-oxodecan-1,11-dioate and 1-iodo-4-phenyl-3-buten-2-one as starting materials. Because of some difficulty in one of the synthetic steps which involved an intramolecular cyclization and the separation of a complex reaction mixture that resulted in this reaction we have directed our efforts towards a new synthetic sequence shown in Scheme 1. This new effort has resulted

Scheme 1



in a very short synthetic sequence to the desired 1. As shown in Scheme 1 we have utilized the known methyl 2-oxocyclopentane heptanoate (2) as the starting material. In this sequence we have found a new method for the introduction of a double bond into cyclopentanone systems (see eg. reaction 2  $\rightarrow$  3 and 4  $\rightarrow$  5). This work has resulted in a recent publication in which we describe the use of cupric bromide as a new method for introduction of double bonds into prostanoid intermediates. This latter process provides one of the shortest sequences to the synthesis of the methyl ester of PGB<sub>1</sub> reported in the literature.

Studies on the Separation of PGB<sub>x</sub>

Separation work was not performed on the PGB<sub>x</sub> complex in the past year of the no cost extension period. The research associate, Dr. G. P. Dhareshwar returned to his native country and there was no point in seeking a replacement at that time, since the large quantities of PGB<sub>x</sub> complex required for our systematic fractionation techniques were not available. Furthermore, the Office of Naval Research was not going to renew our study and it would be fruitless to begin the laborious purification process when sufficient quantities did become available as then the time period was insufficient to accomplish the goal.

APPENDICES

In vitro model of canine cardiac ischemia: alterations in automaticity  
and contractility induced by occlusion<sup>1</sup>

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## INTRODUCTION

A number of important factors have been implicated in the genesis of ischemic heart disease including generalized and focal atherosclerosis, coronary embolism, various forms of arteritis, lupus erythematosus and related connective tissue disorders (8,9). In patients with what appeared to be normal coronary vessels, an abnormality in oxyhemoglobin dissociation (27), acute coronary artery spasm (5,8,30), or an overactive left ventricle, whose excessive oxygen demand cannot be met (1), have also been proposed as contributors to ischemic heart disease. Regardless of the origin, the ultimate cardiac consequences of these disturbances are reflected in irregularities in the functions of the conduction system of the heart (electrical failure) and impaired contractility (pump failure).

Analyses of the discrete changes that are associated with the development of the ischemic state are often confounded by the fact that functional parameters interact with each other and thereby resist definition. In order to characterize the direct impact of ischemia on the functions of selected portions of the dog heart, the following experiments were devised using excised, blood perfused, sinoatrial node (SAN), atrioventricular node (AVN) and papillary muscle (PM) preparations. The susceptibility of these tissues to functional impairment induced by ischemia was also contrasted with that induced by calcium blockade in order to test the hypothesis that the degree of calcium dependence of the tissue is correlated with its vulnerability to oxygen deprivation. It has been proposed (33) that the effects of hypoxia on SAN and AVN are similar to those provoked by the calcium channel blocker, verapamil (36).

## METHODS

Twelve excised sinoatrial node (7,17), 5 atrioventricular node (15), and 16 papillary muscle (12,18) preparations obtained from mongrel dogs of either sex (11.5-15 kg) were used. These isolated tissues were placed in individually water-jacketed chambers maintained at  $38 \pm 0.1^{\circ}\text{C}$  and were perfused through the sinus node artery, anterior and posterior septal arteries, and anterior septal artery, respectively, at a constant pressure of about 120 mm Hg, with arterial blood from the carotid artery of a heparinized supporting dog (15-22 kg) under pentobarbital anesthesia (30 mg/kg, i.v.). A schematic diagram of the system is illustrated in Fig. 1.

The beating rates (automaticity) of SAN, AVN and PM (Purkinje fibers) preparations were measured by a cardi tachometer (Grass, Model 7P4) triggered by the R waves of the electrogram. Developed tension of spontaneously beating or electrically driven papillary muscles was measured by a strain gauge transducer (Grass, FT03C). Rate of blood flow through the PM was monitored by an electromagnetic flow meter (Narco-Biosystems, Model RT500). For some experiments, PM preparations were driven with rectangular pulses of about twice the threshold voltage (0.8-2 V) and 2 msec duration at a frequency of 2 Hz through bipolar platinum electrodes placed diagonally at the base of the tissue (Grass Stimulator, Model S44). All recordings were made on a Grass Model 7D oscillograph.

SKF 24260<sup>4</sup> (Smith Kline & French Laboratories, USA) was first dissolved in Tween-80 (20 mg/ml) and then diluted further with 0.9% NaCl solution to achieve 5 mg/ml (stock solution). Intra-arterial (i.a.) injections of all drug solutions (10-60  $\mu\text{l}$ ) into the preparation were made with micro syringes through the afferent perfusion tubing close to the cannulation point. Total

deprivation of blood supply to the preparations was performed by closing the perfusion tubing with a hemostat.

The measured variables were expressed as the means of N replicates  $\pm$  Standard Error (SE). Significant differences between treatments at specific post-occlusion times were evaluated by Student's unpaired t-test. Significant differences between post-occlusion values for a given treatment and a pre-occlusion control were evaluated by Dunnett's multiple comparison procedure (10).

## RESULTS

Automaticity of SAN preparations. The mean basal sinoatrial rate was  $100 \pm 4.3$  beats/min (N=7). Total occlusion for a period of 30 min caused a clearly defined, time related decrease in sinoatrial rate in all 7 preparations. In 2 preparations the negative chronotropy was preceded by a transient, positive chronotropic response. At 30 min after occlusion, sinoatrial rate decreased by 52 beat/min which was approximately 50% of the pre-occlusion value (Figs. 2A and 2B). Sinoatrial arrest was observed at 20 min in only one of the 7 preparations. After resumption of the blood supply, the decreased sinoatrial rate returned rapidly to the initial pre-occlusion value. Sinoatrial rate just before occlusion (0 min) and at 5 min after reperfusion were not statistically different ( $p > 0.05$ ). Even the preparation that exhibited standstill promptly recovered after the resumption of blood supply; viz. the mean sinoatrial rate at 5 min after the onset of reperfusion was 81 beats/min compared with the preocclusion rate of 96 beats/min ( $p > 0.05$ ).

Automaticity of AVN preparations. The mean basal atrioventricular rate was  $48 \pm 6.4$  beats/min (N=5). The rate decreased uniformly in a

time related manner after deprivation of blood supply as shown in Figs. 2A and 2B. At 30 min after occlusion, atrioventricular rate decreased by 30 beats/min which was approximately 65% of the pre-occlusion value. The AVN rate, like the SAN rate, rapidly returned to pre-occlusion levels after the resumption of blood supply. Unlike the SAN preparation, the AVN exhibited frequent arrhythmias during the 30 min occlusion period which disappeared after reperfusion. This observation regarding the vulnerability of AVN to arrhythmias is essentially consistent with that of Senges et al. on isolated rabbit heart (33).

Automaticity and developed tension of PM preparations. The mean basal rate and developed tension of spontaneously beating papillary muscle preparations were  $45 \pm 4.5$  beats/min and  $3.4 \pm 0.76$  grams, respectively (N=6). A triphasic pattern of automaticity changes followed the total occlusion of blood supply during the 30 min period. This is illustrated in Figs. 2A and 2B. The initial decreasing chronotropism showed a tendency to increase in the direction of the initial preocclusion level during an early 3-10 min period, and this was followed by a rapid and progressive decrease in rate. The contractile force of the PM remained essentially constant for the first few seconds after occlusion and then also rapidly declined (Fig. 3). After 60-90 sec of occlusion, there was a slight increase in contractility lasting less than 2 min and again contractility was progressively diminished. In 5 of 6 preparations, standstill was observed within 13 to 25 min after occlusion. The preparations which exhibited standstill were responsive to electrical stimulation at a frequency of 2 Hz with 2 msec post duration and 1.3 V. These preparations, when stimulated, developed a tension of  $0.3 \pm 0.1$  grams (N=-).

Change in the frequency-force relationship of PM preparations before and after 30 min occlusion. As shown in Fig. 4, canine, blood perfused PM preparations responded well to electrical stimulation and exhibited a positive frequency-force relationship at frequencies up to 3 Hz. At 4 Hz, however, a significant pulsus alternans appeared. After accumulating the data needed to construct a control (pre-occlusion) frequency-force curve, the preparations were paced at a rate of 2 Hz for 10 min and the afferent cannula was clamped. Pre-occlusion tension was  $5.7 \pm 0.66$  grams (N=5). Fifteen minutes after occlusion, tension development was almost 0 (Fig. 3). In paced preparations as well as in spontaneously beating preparations there was a brief partial recovery of contractile activity during the first 1-4 min after occlusion. Within 1 min after reperfusion, significant tension developed which increased rapidly. However, unlike the spontaneously beating PM tissue, recovery of the paced preparation was incomplete 5 min after release. Fifteen min after the resumption of blood supply, a frequency-force relationship was reconstructed. As depicted in Fig. 4, the pulsus alternans was exaggerated and the variation in the alternate tensions at both 3 and 4 Hz were significantly larger than those occurring in the control tissues ( $p < 0.05$ ).

Effects of SKF 24260 on the automaticity in SAN, and automaticity, developed tension in and flow rate through PM preparations. Mean basal SAN rate was  $97 \pm 5.2$  beats/min. Rate and developed tension of spontaneously beating PM were  $46 \pm 4.0$  beats/min and  $4.5 \pm 0.3$  g, respectively. Control blood flow through PM was  $4.9 \pm 0.5$  ml/min.

A single i.a. injection of SKF 24260 (0.3-10  $\mu$ g) into the sinus node artery produced dose-dependent decrease in sinoatrial rate and in the

higher concentrations (3-10  $\mu$ g) it ultimately caused sinoatrial arrest in all 5 preparations (Fig. 5). Arrested tissues began to beat again within 10 min and recovered rapidly. In spontaneously beating PM, SKF 24260 injected into the anterior septal artery, decreased the developed tension and increased the blood flow rate in a dose-related manner. At 300  $\mu$ g, for example, the developed tension decreased by about 90% of control and the blood flow increased to about 120% of control (Fig. 6). The automaticity of PM (Purkinje fibers) on the other hand was only depressed to about 80% of control even at a dose of 300  $\mu$ g.

#### DISCUSSION

The effects of acute anoxia on the mammalian myocardium have been the subject of great interest to both cardiovascular physiologists and pharmacologists, particularly as they relate to development of angina pectoris, myocardial infarction and other ischemic heart disorders. To study the relationship between anoxia and subsequential morphological, functional, and biochemical changes investigators have employed several kinds of preparations; e.g., isolated rat heart perfused with oxygenated Krebs-Henseleit solution (11,16), isolated guinea pig and cat papillary muscle placed in oxygenated physiological solution (6,28,31), isolated rabbit myocardium perfused with oxygenated Tyrode's solution (33), isolated rat and guinea pig heart perfused in a retrograde manner with physiological solution by the Langendorff technique (14,22,23), intact hearts of anesthetized dogs (29), and intact conscious dogs with partially occluded coronary vessels (3,21). Most of these myocardial preparations isolated from cats, guinea pigs, rabbits, rats and dogs have abnormally low beating rates and contractile tensions and they usually could be satisfactorily paced only at 1 Hz or less (6,11,28,31,24). In other words, these hearts were not

working against a physiological stimulus frequency since the average heart rate of cats, dogs, guinea pigs, rabbits and rats are approximately 180-200, 90-100, 280-320, 160-180 and 300-340 beats/min, respectively (unpublished observations). On the other hand, using the blood perfused system described in the present study, SAN firing was  $100 \pm 4.3$  beats/min and PM perfused with blood was able to follow a pacing electrical stimulation as high as 3 or 4 Hz (180 or 240 beats/min; Figs. 2A and 4). It is suggested that these differences between excised blood perfused preparations and those perfused or immersed in physiological solution are better nourished and are operating under more physiological conditions. Furthermore, isolated blood perfused preparations have the advantage of allowing more direct control of most of the major parameters influencing myocardial performance. In this regard, the system's responses may better reflect changes that are likely to occur in vivo.

The automaticity of SAN and AVN exhibited a high degree of resistance to deprivation of blood supply (Fig. 2). SAN, particularly, withstood anoxic insult. Even 30 min after the onset of total occlusion, beating rate was about 50% of control, and in only one of 7 preparations was SAN standstill observed at 20 min. The unique resistance of SAN tissues has also been reported by others working with isolated rabbit hearts (20).

A diminished supply of oxygen and metabolic substrates to a perfused zone of myocardial tissue is known to promote a depletion of glycogen reserves, a decreased rate of ATP production, and a consequent decrease in the tissue concentration of high energy phosphate substances (4,15). Von Kiritshenko (35) and others (26) have observed that the content of glycogen is high in nodal cells and this may be one of the reasons that the SAN or AVN can continue to generate spontaneous electrical activity

for long periods even in the total absence of a blood supply (24).

On the other hand, Purkinje fiber automaticity (Fig. 2) and PM contractility (Fig. 3) were considerably more susceptible to a deprivation of blood supply. Most of these preparations exhibited standstill during a period 13-25 min after the onset of occlusion. The decrease in developed tension to some degree may be associated with the decrease in Purkinje fiber automaticity (PM showed a positive force treppe with increasing stimulation frequency, as might be expected). However, it appears more likely that the decrease in developed tension is due principally to an inability to maintain mechanical contractile force. It can be seen that a deprivation of blood supply caused a significant decrease in developed tension of PM from  $3.4 \pm 0.75$  to  $0.8 \pm 0.02$  (Fig. 3) without a simultaneous change in Purkinje fiber automaticity (Fig. 2) at 10 min post occlusion. Moreover, the preparation which exhibited standstill produced, at best, extremely small contractions in response to direct 2 Hz stimulation (Fig. 3).

Paced papillary muscle tension declined more rapidly during the anoxic period and exhibited a less complete recovery upon resumption of blood supply than was the case for spontaneously beating PM, as might be expected.

Following occlusion, automaticity and contractility of most of the PM preparations exhibited an initial partial recovery of brief duration prior to the more dramatic functional decline. This temporary recovery was observed in 100% of the tissues whose automaticity was monitored and in 74% of the cases in which contractility was monitored. The mechanism underlying this interrupted decline in function cannot be explained at present, although it has been suggested (16) that the mobilization of ATP reserves from specific cell compartments may be responsible.

The beneficial effects of glucose in the external medium or glycogen reserves in tissues are probably essential for restoring not only the contractility but also the automaticity of hypoxic myocardium (20,24,31,33,34). Thus, myocardium exhibiting a positive frequency-force relationship increases its oxygen demand and consumes glycogen reserves at a rate correlated with the stimulation frequency. It is especially interesting that extremely depressed contractility which was observed during the occlusion period was not irreversible although its recovery was slower than that which characterized the automaticity parameter. After the resumption of blood supply, developed tension was restored to about 95% of the control value within 15 min. However, the frequency-force curve obtained at 5 min post-occlusion clearly indicated that the contractile machinery per se had not completely recovered. The myocardial tissues once subjected to anoxic stress exhibited a lack of functional reserve.

As a result of the restriction of coronary blood flow electrolyte shifts, such as an increase in tissue sodium and calcium ions and a loss of sodium ion, are known to occur (19,23). To what extent such distortion of ion distribution participates in the response to ischemia may, in part, be determined by examining the response of tissues to ion changes in the absence of anoxic stress. The administration of SKF 24260, a calcium channel blocking drug, significantly depressed SAN automaticity and PM contractility without provoking a comparable reduction in Purkinje fiber automaticity. These results are consistent with those reported by Endo et al. (13) who were investigating two other calcium antagonists, verapamil and Bay 1040. Thus, these observations may fortify further the concept that in Purkinje fibers, unlike SAN and AVN, an outward potassium current may account for the generation of pacemaker depolarization (13,25). It is well established

that the slow calcium-carrying system sensitive to calcium blocking drugs plays an important role in generating the action potential of SAN and AVN (17,36,37,38) rather than the fast sodium-carrying system sensitive to tetrodotoxin. In contrast, the electrical activity of myocardial muscle fibers is dependent mainly on a fast inward sodium current (2,32,37). Since the parameters most vulnerable to the deprivation of blood supply were automaticity of Purkinje fibers and contractility of ventricular muscle and the least vulnerable was automaticity of SAN, it might be generalized that in the conduction system the more distal to the sinoatrial node, the more vulnerable to hypoxia. It also seems reasonable to suggest that acute deprivation of blood supply does not predominantly damage the functions of tissues in which the slow inward current is initiated by calcium ions. This conclusion is incompatible with that presented by Senges et al. (33) in experiments with isolated rabbit myocardium. At least part of the discrepancy could be explained by the fact that differences exist in a) the experimental method; blood perfusion via an accessible artery versus superfusion with artificial physiological solution, b) the animals used in the study; dogs versus rabbit and c) experimental protocol; total deprivation of blood supply versus hypoxia induced by substituting nitrogen gas for oxygen.

The responses of canine blood perfused tissues, we believe, more closely approximate in vivo functionality, and to this extent are convinced that these preparations are useful for studying myocardial responses to physiological, biochemical, metabolic and pharmacological assaults.

## FOOTNOTES

<sup>1</sup>Supported by contract N00014-79-C-0122 from the Office of Naval Research.

A preliminary account of these experiments appeared in abstract form in Fed. Proc. 40:692, 1981.

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<sup>4</sup>1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridine dicarboxylic acid diethylester.

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Fig. 1. Schematic diagram of the isolated blood-perfused cardiac tissue system. Each of the three tissue types are independently mounted in water-jacketed chambers and perfused through an accessible artery with blood from an anesthetized supporting dog.

Fig. 2. Spontaneously beating sinoatrial node (●), atrioventricular node (▲) and Purkinje fiber (■) preparations during, prior and after total deprivation of blood supply. Horizontal bar indicates the period of occlusion. Data points are means  $\pm$  SE of 5-6 preparations in each tissue category. Panel B presents the Panel A responses as percents of pre-occlusion control values. Starred points are significantly different from their respective control ( $p < 0.05$ ).

Fig. 3. Contractility of spontaneously beating papillary muscle (●) and papillary muscle paced at a frequency of 2Hz (▲) prior, during and after total deprivation of blood supply. Open circle represents the mean response of 5 tissues (that exhibited spontaneous stand-still) to 2 Hz stimulation. Other graphic notations similar to those in Fig. 2.

Fig. 4. Frequency-force curves for papillary muscle preparations (N=5) 20 min prior to occlusion (○) and 15 min after reperfusion (●). Data points are means  $\pm$  SE. The "Auto" points indicate spontaneous rates of firing in the absence of external stimulation.

Fig. 5. Spontaneous rate of firing of SAN preparations in the absence (○) and presence (●) of varying concentrations of SKF 24260 injected into the afferent arterial cannula. Data points represent means  $\pm$  SE of 1-5 tissues.

Fig. 6. Spontaneous rate of firing (A), contractility (B) and blood flow (C) in papillary muscle preparations in the absence (○) and presence (●) of SKF24260 injected into the afferent arterial cannula. Data points represent means  $\pm$  SE of 5 tissues.

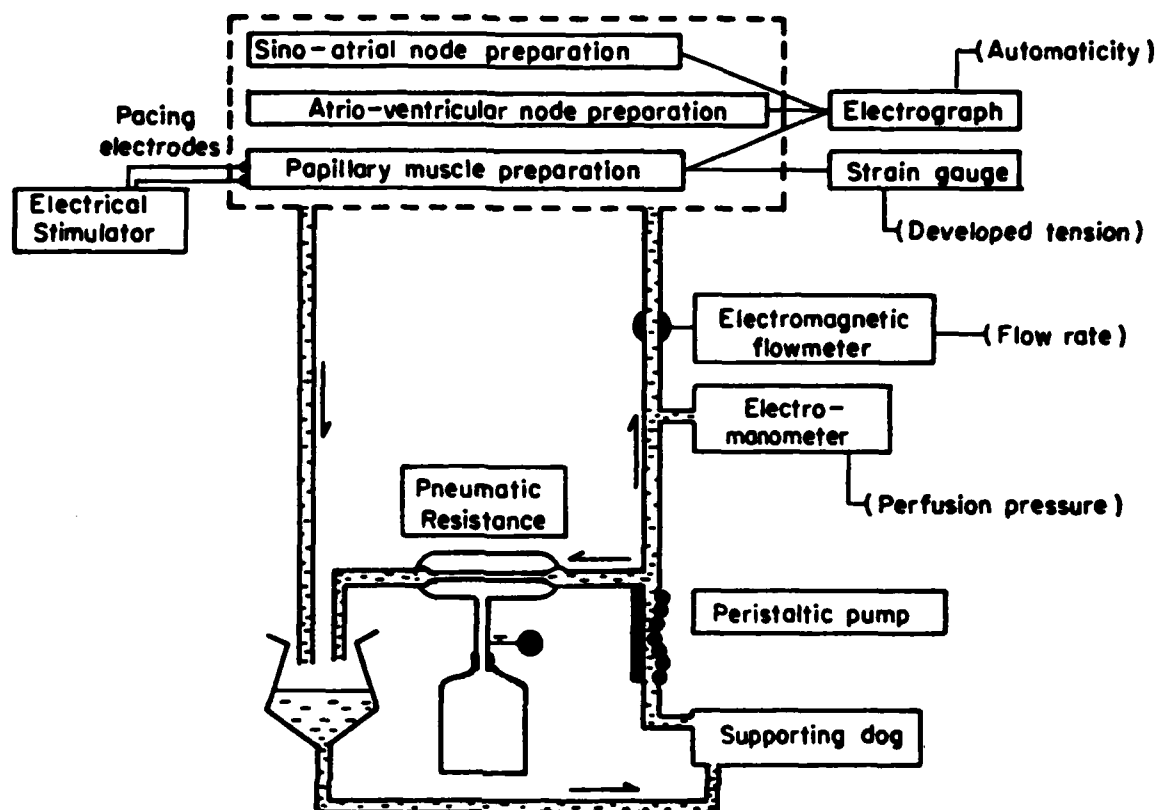


Figure 1

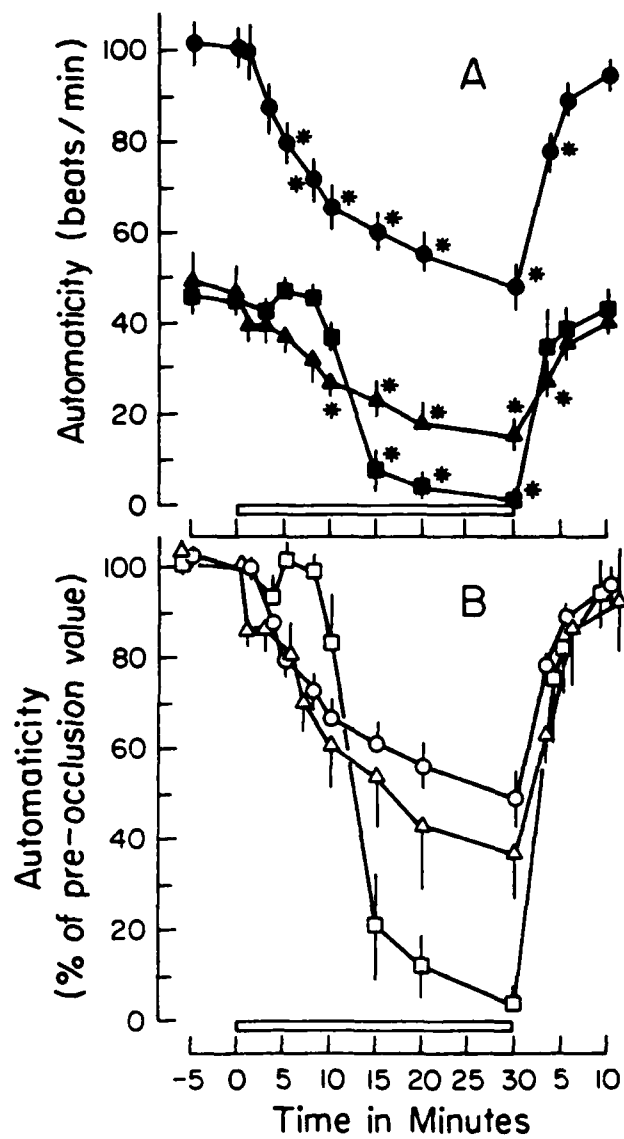


Figure 2

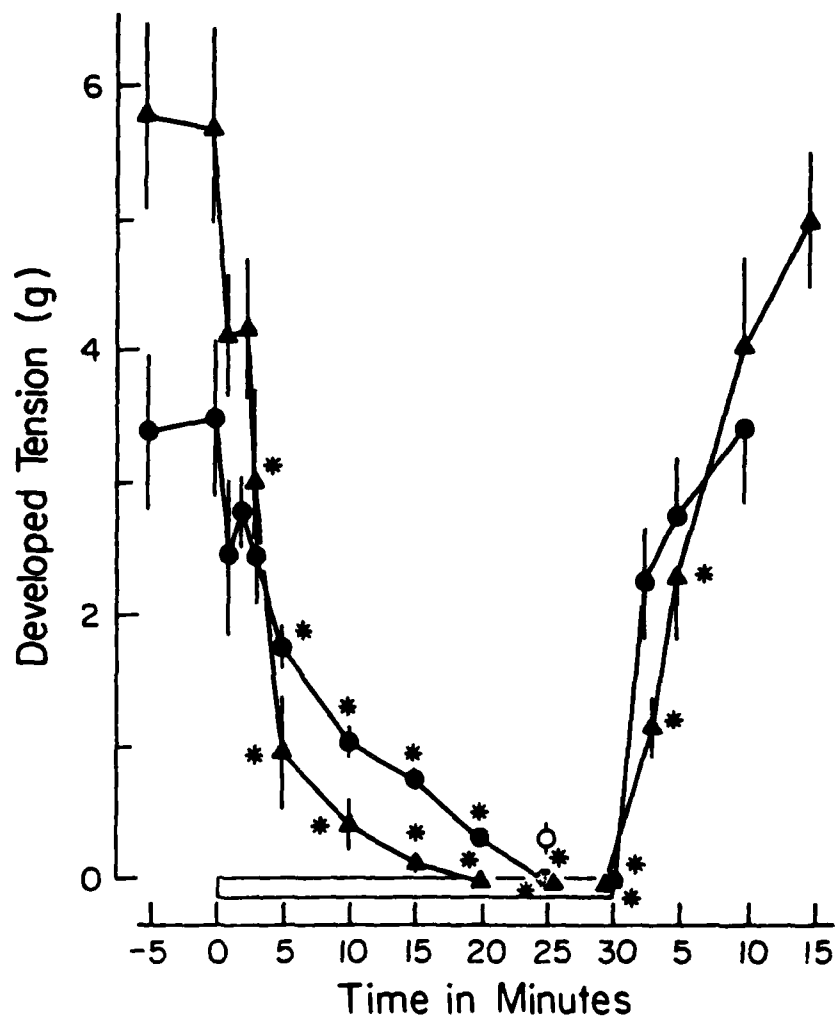


Figure 3

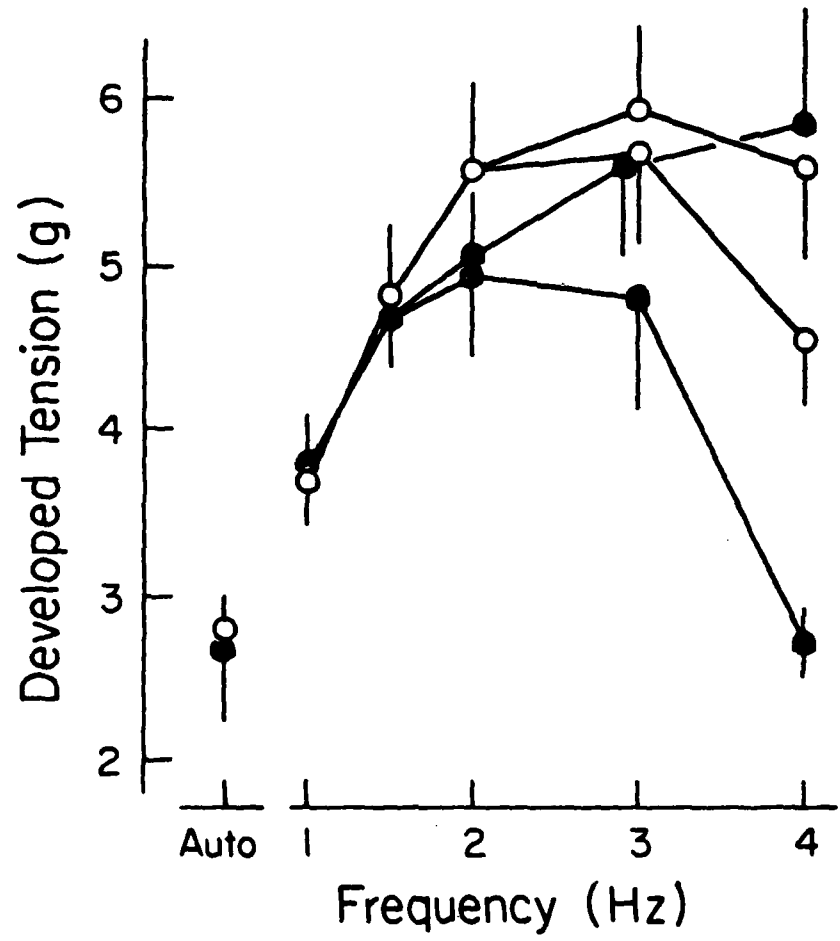


Figure 4

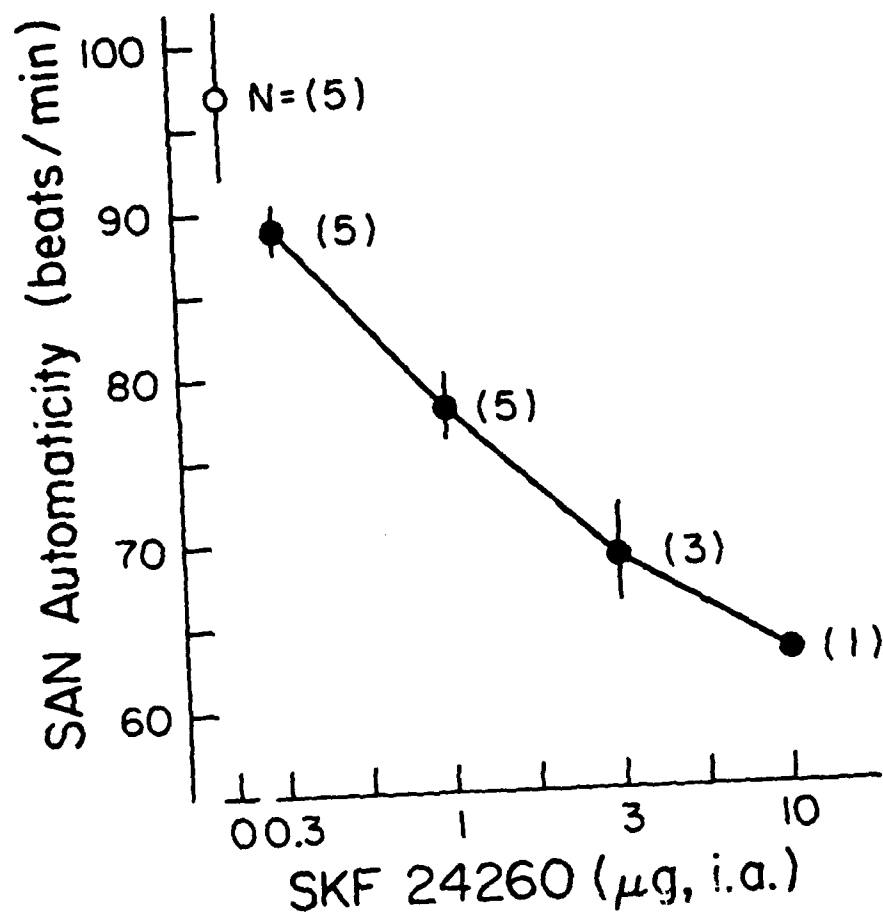


Figure 5

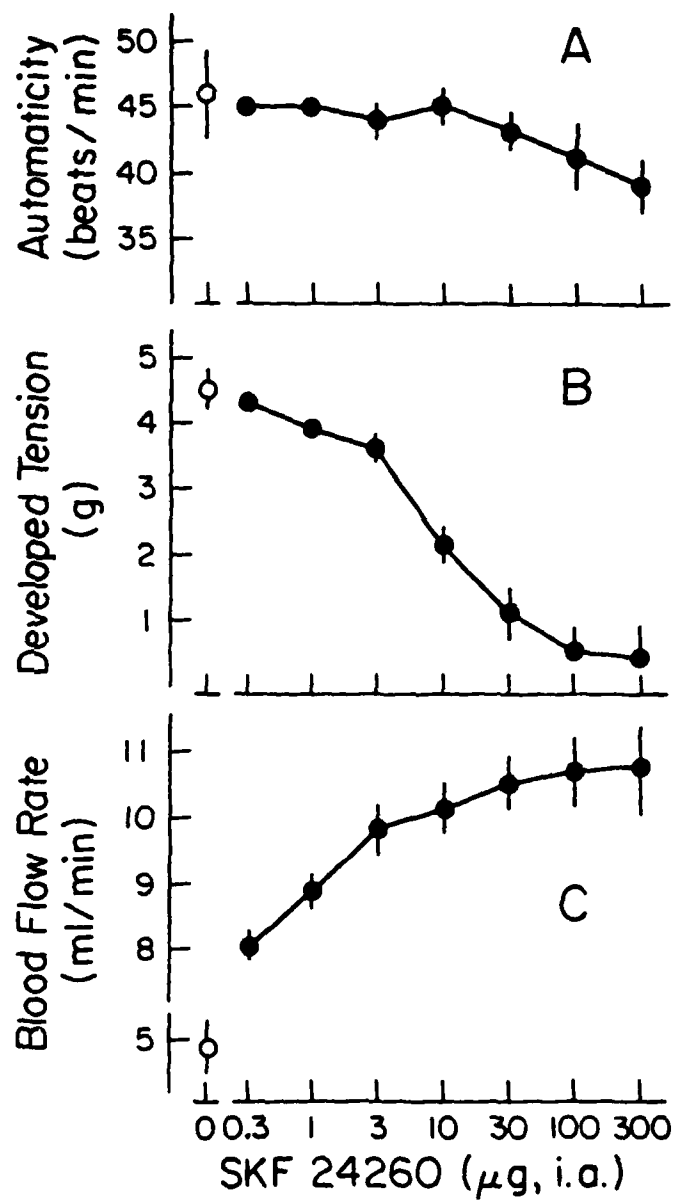


Figure 6

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## APPENDIX B

### ACUTE TOXICITY OF PROSTAGLANDIN Bx IN MALE, ALBINO, ICR MICE

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## ABSTRACT

Median lethal doses were estimated for PGBx administered to mice by the intraperitoneal and intravenous routes. The incidence of lethality (and therefore the LD50) was time dependent over a 96 hour period. The animals were active, were responsive and took nourishment during the 4-day post-injection period so that they apparently died from the direct effects of PGBx (or its metabolites) and not as a consequence of depression-induced dehydration or starvation.

## INTRODUCTION

Prostaglandin Bx (PGBx) is a complex mixture of oligomers derived from the base-catalyzed polymerization of 15-keto-PGB, methyl ester (Polis *et al.*, 1979a). It has been reported to exert effects on a variety of biological systems including the a) phosphorylating ability of age-degraded rat liver mitochondria (Polis *et al.*, 1973; Polis *et al.*, 1979b), b) short term survival of monkeys with induced myocardial infarctions (Angelakos *et al.*, 1980), c) inotropic response of anoxic canine papillary muscle (Walls *et al.*, 1981), d) autonomic cardiopulmonary activity during cerebral naoxia (Moss *et al.*, 1978).

e) peripheral vascular response to sympathetic stimulation (Himori and Burkman, 1980) and f) body weight and blood glucose levels of diabetic (Polis and Polis, 1976) and genetically obese, non-diabetic mice (Polis and Cope, 1980).

The concentrations of PGBx that were observed to provoke measurable responses in in vivo systems (summarized in Table 1.) vary over a considerable range and differences in routes of administration, dosing regimens and animal subjects make it virtually impossible to determine the true breadth of the dose range over which this material might be pharmacologically active and, more importantly, the concentrations that approach levels that threaten the life of the animal.

In an attempt to establish upper dosing limits for PGBx (in one species) the acute median lethal doses were estimated by two routes of administration and time profiles of the lethal effect were constructed.

#### MATERIALS AND METHODS

Animals. Male albino ICR mice (Hap:Swiss(ICR)BR), 18-22 g were housed in a temperature (22-24°C) and light controlled (0600-1800) vivarium in plastic cages (10/cage) with processed corn cob bedding. The animals were fasted 4 hours prior to the experiment but allowed continuous access to water during this period.

PGBx preparation. PGBx was supplied by Dr. H.W. Shmukler, (Biochemistry Branch, Naval Air Development Center, Warminster, PA) as a dark amber ethanolic solution containing 155.6 mg of PGBx acid/ml.\* It was converted to the water soluble sodium salt by using a method similar to that described by Kolata and Polis (1980), freeze dried and stored in evacuated ampules at 5°C. Sodium PGBx solutions, having a pH of 7.6, were reconstituted as needed with normal saline or water (depending upon the solute concentration desired) and passed through a cellulose triacetate membrane filter (0.2 micron nominal pore size) into sterile, stoppered, serum vials. All doses described in this report are

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\*This sample was described by H.W.S. as Fraction 2 (Sephadex LH20 column separation) of preparation No. 28.

Table 1. Estimation of PGBx doses used in vivo studies.

Dose of PGBx <sup>a</sup>	Animal	Citation
1 mg/kg, IM <sup>b</sup> + 1 mg/kg, IC <sup>c</sup> + 1 mg/kg, IV <sup>d</sup> +	Rhesus monkeys African green monkeys	Angelakos <i>et al.</i> (1980)
8-10 mg/kg/day, SC <sup>e</sup>	Diabetic mice (C57BL/KsJ-db)	Polis & Polis (1976)
5-20 mg/kg/day, IM <sup>f</sup>	Obese mice (57BL/6J-ob)	Polis & Cope (1980)
1 mg/kg, IV <sup>g</sup>	New Zealand rabbits	Kolata & Polis (1980)
1 mg/kg x 3, IV + 0.5 mg/kg x 4, IV <sup>h</sup>	Beagles	Moss <i>et al.</i> (1978)
2.4-12 mg/kg/day, SC <sup>i</sup> , 10 mg/kg, IV	Sprague Dawley rats	Himori & Burkman (1980)

<sup>a</sup> Authors of the cited studies did not always make clear the form of PGBx that was used but it is assumed that the doses expressed are in terms of the water soluble sodium salt of PGBx.

<sup>b</sup> Intramuscular route; given prior to coronary ligation.

<sup>c</sup> Intracardiac route; given post-ligation.

<sup>d</sup> Intravenous route; dose was repeated every 30 minutes.

<sup>e</sup> Subcutaneous route; given 5 times each week for up to 24 weeks. Only total doses are reported by the authors and doses/kg were estimated from mouse weights.

<sup>f</sup> Given 5 times each week for up to 18 weeks.

<sup>g</sup> Authors do not explicitly indicate route of administration but from the nature of the preparation, the intravenous route was inferred.

<sup>h</sup> Total dose was 5 mg/kg.

<sup>i</sup> Given 7 days. Dose was expressed in terms of PGBx acid.

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expressed in terms of PGBx acid.

Quantal lethality determine. Groups of mice (8-12) received graded doses of sodium PGBx by either the intravenous (IV) or intraperitoneal (IP) route, placed in individual observation chambers along with food and water and were examined continuously during the first 2 hours post-injection and at periodic intervals thereafter until it was clear that no additional deaths occurred. Data describing the relation between incidence of lethality and time from injection were collected and median lethal doses (LD50's) of PGBx and their standard errors (SE's) were estimated for several time periods according to the method of Miller and Tainter (1944). Each LD50 was interpolated from a curve to which 29-51 mice had contributed data.

Gross behavioral activity and tests for neurological deficit. Using the techniques and protocols of Irwin (1964) and Dunham and Miya (1957), mice receiving varying IP doses of sodium PGBx (6 mice/dose) were periodically examined for gross signs of performance impairment during a 24 hour post-injection period.

#### RESULTS AND DISCUSSION

Samples of sodium PGBx were submitted to Dr. T.M. Devlin (Department of Biological Chemistry, Hahnemann Medical College, Philadelphia, PA) for assay in order to assess the potency of the preparation against a reference standard PGBx used by Devlin and his colleagues in their in vitro studies (e.g., Ohnishi and Devlin, 1979). This assay measured the ability of PGBx to increase the in vitro esterification of phosphate by suspensions of rat mitochondria whose oxidative phosphorylating capacity had been purposefully impaired (Polis, et al., 1979). The results, depicted in Table 2, indicate that the sample is virtually indistinguishable from the reference standard. I am satisfied, therefore, that the sample has a potency comparable with that of PGBx used by others.

The LD50's interpolated from log dose-probit response curves, were clearly time-dependent (Table 3) during the first 96 hours

post-injection. Animals rarely died during the first 6 hours (regardless of the route of administration) and the full lethal consequences of PGBx were not seen until 4 days had elapsed. One might speculate that the organ damage that caused death developed slowly or that the lethal determinant was a slowly accumulating metabolite of PGBx that required a relatively long period of time to reach a lethal concentration.

Figure 1 illustrates the time dependency profile of both the IV and IP routes. Animals remained active, responsive to external stimuli and

**Table 2.** Mitochondrial Oxidative Phosphorylation Assay of the Sodium PGBx Sample (-1AMB) Compared with the Reference Standard (-RS).

Sample	Change in $\mu\text{M}$ Phosphate Esterified (Mean $\pm$ SE) <sup>a</sup>
PGBx-Na-RS (38 $\mu\text{g}$ ) <sup>b</sup>	18.2 $\pm$ 0.9
PGBx-Na-1AMB (38 $\mu\text{g}$ )	17.2 $\pm$ 1.9
1AMB as % of RS	94.7

<sup>a</sup>Mean of triplicate determinations.

<sup>b</sup>Expressed as the quantity of PGBx acid/2.8 ml reaction mixture.

**Table 3.** Acute lethality of Sodium PGBx in Male ICR Mice.

Post-Injection Time (hrs)	Median Lethal Dose $\pm$ SE (mg/kg) <sup>a</sup>	
	Intravenous Route	Intraperitoneal Route
24	180 $\pm$ 26 (29) <sup>b</sup>	400 $\pm$ 41 (39)
48	91 $\pm$ 8 (41)	170 $\pm$ 30 (51)
72	82 $\pm$ 6 (35)	140 $\pm$ 30 (51)
96	69 $\pm$ 6 (35)	90 $\pm$ 27 (51)

<sup>a</sup>Expressed as PGBx acid.

<sup>b</sup>Number of mice contributing to the log dose-lethal incidence curve from which the corresponding LD50 was interpolated.

took nourishment during the 4-day post-injection period so that I believe their death was a result of the "direct" effect of PGBx (or its metabolites) and not as a consequence of depression-induced dehydration or starvation.

Assessment of behavioral and neurological behaviors (Irwin, 1964; Dunham and Miya, 1957) revealed that single IP doses of PGBx in excess of 25 mg/kg (equal to about 30% of the 96-hr LD50) produced a reduction of alertness, spontaneity and reactivity. Some loss of abdominal motor tone was seen and locomotor movements tended to be uncoordinated and spastic during the first 24 hrs. Although mice remained fully conscious and active, their behavior (using a variety of Irwin's behavioral paradigms) was generally sluggish when compared with controls. Doses less than 25 mg/kg, IP, produced virtually no acute abnormal behavioral deficits.

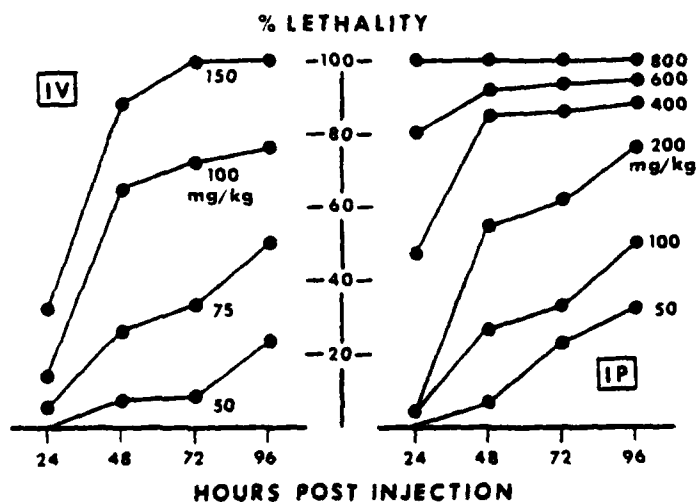


Figure 1. Time-effect curves for PGBx-induced lethality in male albino ICR mice for various concentrations administered by the intravenous (IV) or intraperitoneal (IP) routes. The incidence of death is maximum for all doses at 96 hours. N = 29-51 mice/curve.

#### ACKNOWLEDGEMENT

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PROSTAGLANDIN B<sub>1</sub> CAN MODIFY THE PRESSOR RESPONSES  
TO SYMPATHETIC NERVE STIMULATION

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## ABSTRACT

PGB<sub>1</sub>, a metabolite of PGA<sub>1</sub>, has the ability to enhance peripheral vascular resistance and elevate blood pressure in animals whose tone is low. The effect is not central in origin and apparently is not the result of changes in alpha adrenoceptor sensitivity or changes in vascular smooth muscle susceptibility per se.

## INTRODUCTION

It has been proposed that prostaglandins (PG's) play important roles as mediators and modulators of a variety of physiological and pathological functions, for example, in the regulation of neurotransmitter release from sympathetic nerve endings (PGE's and PGF<sub>2α</sub>) (1,2), in the prevention of intra-arterial thrombus formation (PGI<sub>2</sub>) (3), in the induction of bronchospasm (PGF<sub>2α</sub>) (4) and inflammation (PGE's) (5), and in the control of electrolyte balance (PGA<sub>2</sub> and PGE<sub>3</sub>) (6). However, it is generally assumed that prostaglandins of the B series are almost devoid of activity on the cardiovascular system (7) and in the literature known to us there is little information on the cardiovascular effects of PGB<sub>1</sub> (8). In the present study we demonstrate that in pithed rats, whose sympathetic tone is very low, PGB<sub>1</sub> selectively potentiates the pressor responses induced by both endogenous and exogenous norepinephrine.

## METHODS

In one series of experiments male Wistar rats (260-305 g) were prepared, with minor modification, according to the method of Gillespie et al. (9). Rats which had been atropinized were anesthetized with ether, adrenalectomized, pithed and artificially ventilated. A stimulating electrode was introduced into vertebral canal via the right orbit and an indifferent electrode was inserted

through the dorsal skin surface. The stimulated region was at the level of  $T_9 - L_1$ . Tubocurarine, i.v., was administered and sub-maximal 1 msec, 20 volt pulses were delivered at graded frequencies (0.3-10 Hz) for 14 sec periods at 2 min intervals. Systolic blood pressure was directly measured from the femoral artery (Statham P23 ID transducer) and drugs were administered via a catheter inserted in the femoral vein.

PGB<sub>1</sub> methylester was dissolved in an ethanol-pH 7.4 phosphate buffer solution (55:45). The vehicle alone was also examined for its influence on the cardiovascular system and served as a solvent control.

## RESULTS AND DISCUSSIONS

In these pithed animals bolus i.v. injections of PGB<sub>1</sub> (10 mg/kg) significantly increased the basal systolic and diastolic blood pressure from  $54 \pm 3.3/41 \pm 3.4$  to  $116 \pm 2.6/88 \pm 2.9$  mm Hg ( $p < 0.001$ ) without substantially altering the heart ( $311 \pm 9.8$  to  $321 \pm 11.2$  beats/min,  $p > 0.3$ ). The changes in blood pressure slowly diminished and disappeared within 1.5 hr. After i.v. bolus injection of PGB<sub>1</sub> the pressor response to electrical stimulation of sympathetic outflow was significantly augmented in a time dependent manner. The maximum effect was seen at 30 min post-injection (Fig. 1). When the changes were expressed on a percent basis, the pressor responses to lower frequencies (0.3-0.8 Hz) were potentiated to a greater extent ( $p < 0.01$ ) than were responses to higher frequencies (5-10 Hz).

In unstimulated, adrenalectomized, pithed rats PGB<sub>1</sub> also potentiated the pressor responses to i.v. (-)-norepinephrine but failed to significantly modify the pressor responses to angiotensin II (Fig. 2A) or to methoxamine, an alpha-adrenoceptor stimulant (Fig. 2B). Norepinephrine was about 0.3 times as active as angiotensin II in these animals. PGB<sub>1</sub> caused a fourfold shift to the left of the norepinephrine curve (at doses of 10 mg/kg) and began to express its pressor enhancing actions at doses as low as 0.5-1 mg/kg.

On the other hand, PGB<sub>1</sub> failed to exhibit a pressor enhancement effect in pentobarbital anesthetized rats that had not been subject

<sup>1</sup>An abstract of this work appeared in The Pharmacologist 22:256, 1980.

to pithing or adrenal ablation. Moreover, in these animals (whose sympathetic tone was substantially higher than that of pithed rats)  $\text{PGB}_1$  did not enhance the pressor response to norepinephrine (Fig. 3B). These male Wistar Rats (280-320 g) had systolic pressures and heart rates of  $133 \pm 8.9$  mm Hg and  $394 \pm 13.8$  beats/min (mean  $\pm$  s.d.,  $n=27$ ), respectively.  $\text{PGB}_1$  also failed to significantly alter the vasodepressor response to a) acetylcholine (Fig. 3A) and to b) vagal stimulation<sup>2</sup>.

It appears that the vascular effects of  $\text{PGB}_1$  are specifically related to the functions of the sympathetic system and that these effects are expressed clearly only when sympathetic tone is low and when small changes in norepinephrine concentrations in the vicinity of the alphaadrenoceptors is expected to produce dramatic changes in vascular tone. It is suggested that the potentiating action of  $\text{PGB}_1$  may be mediated by an interference with the reuptake of catecholamines and/or their metabolism by monoamine oxidase or catechol-O-methyltransferase. Although it is less likely, it is also possible that  $\text{PGB}_1$  facilitates transmitter release from sympathetic neurons. It seems unlikely that the action is due to an increase in alpha-adrenoceptor sensitivity or to enhanced changes in vascular smooth muscle responsiveness, per se. It is clear that the effects are not central in origin.

It is known that  $\text{PGC}_1$  and  $\text{PGC}_2$  which are converted to  $\text{PGA}_1$  and  $\text{PGA}_2$  by mammalian prostaglandin isomerase are unstable compounds and are metabolized to  $\text{PGB}_1$  and  $\text{PGB}_2$ , respectively (10,11).  $\text{PGA}$  compounds exert an antihypertensive effect by reducing total peripheral resistance including the resistance of renal arterioles (12,13), an effect which is particularly striking in some hypertensive patients (14). It is curious that  $\text{PGB}_1$ , a metabolite of  $\text{PGA}_1$ , has, under conditions of low sympathetic tone, the apparent capacity to elevate vascular sympathetic tone. We wonder to what extent endogenous  $\text{PGB}_1$  may influence the blood pressure and the functions of other organs innervated by the sympathetic nervous system.

<sup>2</sup> Femoral arterial blood pressure was monitored in pentobarbital anaesthetized rats (60 mg/kg, i.p.) whose right vagi were sectioned. The peripheral segment was stimulated with 2 msec pulses of approximately 20 volts and frequencies of 10 and 20 Hz for 20 sec periods at 4 minute intervals (Grace model S44 stimulated).

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### Figure legends

Fig. 1 Pressor response curves from spinal cord stimulated, adrenalectomized, pithed rats before and 30 min after  $\text{PGB}_1$  (10 mg/kg i.v.) or its vehicle. Each point represents a mean  $\pm$  s.e. of n experiments.

Fig. 2 Pressor response curves from adrenalectomized, pithed rats receiving graded doses of noradrenaline (NA), angiotensin II (Ang II) (Panel A) and methoxamine (Metho) (Panel B) before and 30 min after  $\text{PGB}_1$  (10 mg/kg i.v.) or its vehicle. Each point represents a mean  $\pm$  s.e. of n experiments.

Fig. 3 Changes in vasodepressor response curves (A) to acetylcholine (ACh) and in pressor response curves (B) to noradrenaline (NA) in pentobarbitone anaesthetized rats before and 30 min after  $\text{PGB}_1$  (10 mg/kg i.v.). Each point represents a mean  $\pm$  s.e. of n experiments.

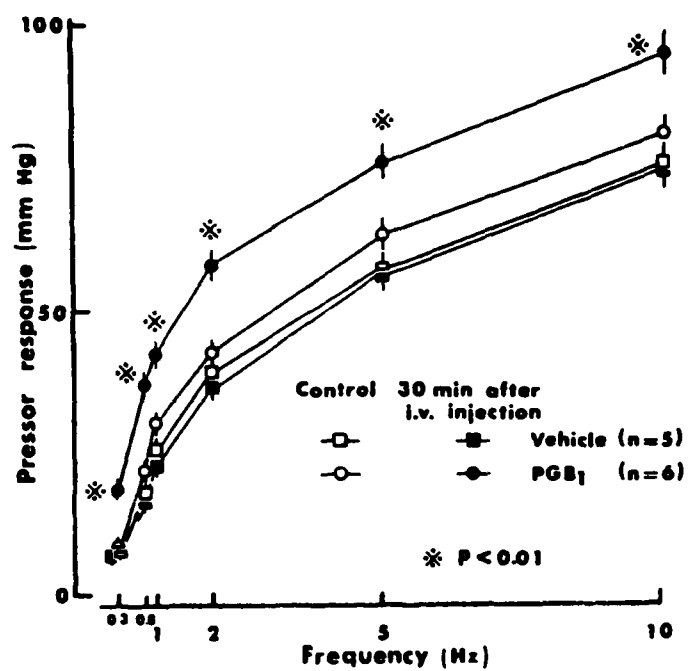


Figure 1

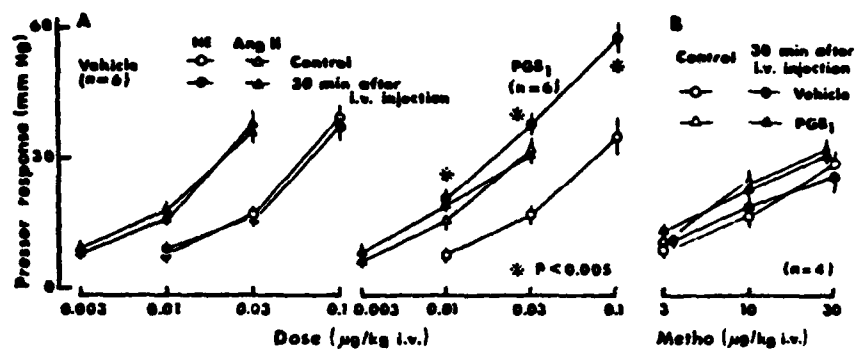


Figure 2

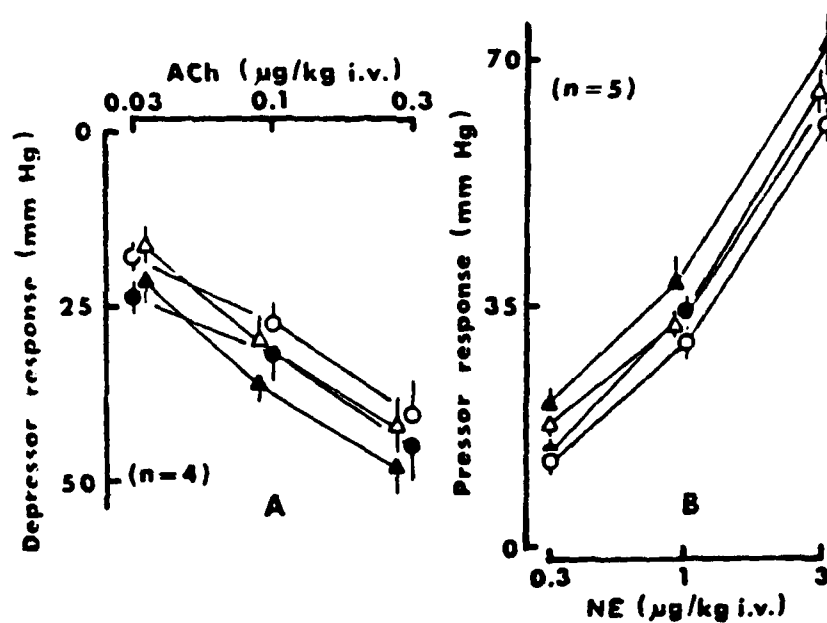


Figure 3

APPENDIX D

Table 1

Influence of PGBx on ouabain-induced cardiotoxicity in guinea pigs.

Drug	Dose <sup>a</sup>	N	Concentration of ouabain producing <sup>b</sup>		
			Arrhythmia	Fibrillation	Cardiac Arrest
Vehicle	-	7	9±0.3 <sup>c</sup>	14±0.9	18±1.3
PGBx	0.1	4	8±0.3	13±1.1	16±1.3
PGBx	1.0	3	10±0.6	15±1.7	18±1.2
PGBx	10.0	5	11±0.7*	17±1.0*	21±1.1

<sup>a</sup>mg/kg, IV<sup>b</sup>mcg/kg, IV<sup>c</sup>Mean ± SEM\* Significant at  $p < 0.05$ 

Note: The higher the concentration needed to produce a symptom of toxicity, the lower the apparent toxic potency of ouabain.

## APPENDIX E

### CUPRIC BROMIDE UTILIZATION IN THE SYNTHESIS OF PROSTANOID INTERMEDIATES

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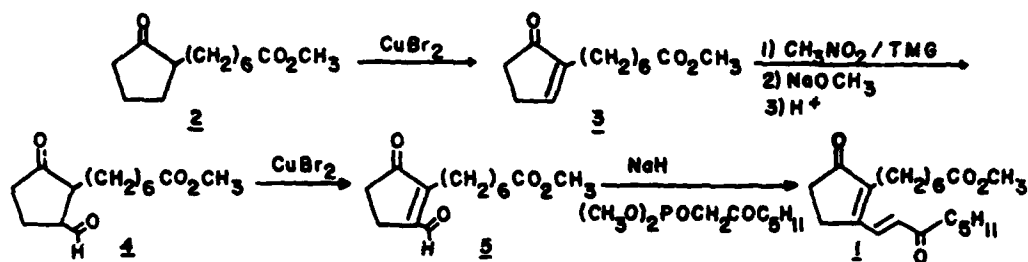
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**Abstract:** An effective, one-step procedure using  $\text{CuBr}_2$  is reported for the introduction of a double bond into the prostanoid nucleus.

Prostaglandin  $\text{B}_x$  ( $\text{PGB}_x$ ) is a new oligomeric derivative of prostaglandin  $\text{B}_1$  ( $\text{PGB}_1$ ) which is prepared from the methyl ester of 15-keto- $\text{PGB}_1$ .<sup>1,2</sup>  $\text{PGB}_x$  has been reported to restore oxidative phosphorylation of isolated degraded rat liver mitochondria. Considerable effort has been directed towards the isolation and characterization of components of  $\text{PGB}_x$ .<sup>1,2,3</sup> We have been investigating new shortened synthetic sequences to the methyl ester of 15-keto- $\text{PGB}_1$ .<sup>2,4</sup> (1) that would provide large quantities of this material for the eventual preparation of  $\text{PGB}_x$ . In this work we found an unusual reaction of cupric bromide that allows for a one-step introduction of a double bond in the prostanoid cyclopentane ring system.

In a number of approaches to prostanoids, methyl or ethyl 2-oxocyclopentane heptanoate (2) has been reported as an important intermediate.<sup>5</sup> Dropwise addition of 5.7g (25.2 mmol) of 2 in 30 ml of  $\text{CHCl}_3$  was carried out over a 10 min. period to a refluxing suspension of 12g cupric bromide (53.7 mmol, anhydrous, 99%, Aldrich Chemical Co.) in anhydrous ethyl acetate (30 ml) with vigorous stirring. The stirred solution was allowed to reflux for an additional 30 min. until the color of the reaction mixture changed from green to amber. After cooling to room temperature and filtration, the solvent was removed to give a residue which was dissolved in ether (50 ml) and washed with brine (50 ml). The organic layer was dried ( $\text{MgSO}_4$ ), treated with charcoal and evaporated to give an oil that was purified by chromatography on silica gel using  $\text{CH}_2\text{Cl}_2$  as the eluent to afford 3.6g (66% yield) of the cyclopentenone (3). We found this one-step procedure to be superior to the multiple-step procedure recently reported by Bernady and co-workers<sup>6</sup> for the preparation of 3. The conversion of 3 to 4 was carried out according to the procedure of Bagli and Bogri.<sup>7</sup> The same cupric bromide procedure was applied to keto aldehyde 4 and it afforded a 38% yield of the

desired product 5. The reaction of 5 with dimethyl 2-oxoheptyl phosphate in the presence of sodium hydride gave the desired methyl ester of 15-keto-PGB<sub>1</sub> (1).<sup>8</sup>



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